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(57) Abstract

Novel modified receptor proteins and ligands that bind to these receptor proteins are provided. Also included in the present invention are methods of identifying ligands that bind to such modified receptor proteins and methods of use of the modified receptor to modulate the expression of an exogenous gene in a mammalian subject.

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METHOD FOR IDENTIFICATION OF MODIFIED RECEPTOR LIGANDS AND METHODS OF USE THEREFOR

BACKGROUND OF THE INVENTION

1. *Field of the Invention*

This invention relates generally to ligand-receptor interactions and more specifically to modified receptor proteins and ligands which bind to such receptors and methods of use therefor.

2. *Description of Related Art*

The study of receptor-ligand interactions has revealed a great deal of information about how cells respond to external stimuli. This knowledge has led to the development of therapeutically important compounds, such as erythropoietin, colony stimulating factors and PDGF.

Although much remains to be discovered regarding the molecules and mechanisms that mediate specific gene regulation in response to exogenous inducers, it is known that exogenous inducers modulate gene transcription by acting in concert with intracellular components, including intracellular receptors and discrete DNA regions known as response elements.

It is known that once hormones, such as glucocorticoid, enter a cell, they bind to specific receptor proteins, thereby creating a ligand/receptor complex. The binding of the hormone to the receptor is believed to initiate an allosteric alteration of the receptor protein. As a result, it is believed that the ligand/receptor complex is capable of binding with high affinity to certain specific sites on the chromatin DNA. Such sites, which are known as response elements, modulate expression of nearby target gene promoters.

Modulators of transcription factors involved in direct regulation of gene expression have been described, and include those extracellular substances entering the cell passively and binding with high affinity to their receptor-transcription factors. This class of direct t-ranscriptional modulators include steroid hormones and their analogs, thyroid hormones, retinoic acid, and vitamin D3 and its derivatives.

Pharmaceuticals which increase or decrease the expression of genes will have important clinical application for the treatment of a variety of diseases and conditions. The clinical use of steroid hormones, thyroid hormones, vitamin D3 and their analogs demonstrates that agents which modulate gene transcription can be used for beneficial effects. Analogs of these agents that do not bind to the wild-type receptor, but bind to a modified receptor linked to an exogenous gene, such as a therapeutic gene, could have clinical utility.

SUMMARY OF THE INVENTION

The present invention is based on modification of receptor proteins which function as ligand-dependent transcription factors for use as therapeutic agents. In a first embodiment, the invention provides a method for identifying a functional ligand for a modified receptor protein, the method comprising a)introducing into a suitable host cell, a nucleic acid encoding a modified receptor protein and an exogenous gene functionally linked to a modified response element capable of activation by the modified receptor protein wherein the modified receptor protein has a modified ligand-binding domain and a modified DNA binding domain which render it incapable of transactivating genes transactivated by the corresponding wild-type receptor; b)contacting the cell containing the modified receptor protein and exogenous gene with test ligand(s); c) monitoring expression of the exogenous gene; and d) identifying the ligand.

In another embodiment, the invention provides a method for modulating the expression of an exogenous gene in a mammalian subject containing a)a DNA construct comprising the exogenous gene under the control of a modified response element; and b)a modified receptor protein which in the presence of a ligand therefor, binds to the modified response element the method comprising administering to the subject an effective amount of a ligand for the modified receptor protein; wherein the ligand is not normally present in the cells of the subject and wherein the ligand differs from a ligand that binds to the wild-type receptor.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides materials and methods that are useful for identifying, isolating and preparing ligands for modified receptor proteins and polynucleotide molecules encoding those ligands. The present invention thus provides useful tools for identifying new ligands and receptors that are useful in research, studies of cell physiology and metabolism, and therapeutic intervention in animals, including humans.

In the description that follows, a number of terms used in the field of ligand-receptor interactions and recombinant DNA technology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, definitions are provided.

In a first embodiment, the invention provides a method for identifying a functional ligand for a modified receptor protein. The method includes introducing into a suitable host cell, a nucleic acid encoding a modified receptor protein and an exogenous gene functionally linked to a modified response element capable of activation by the modified receptor protein wherein the modified receptor protein has a modified ligand-binding domain and a modified DNA binding domain which render it incapable of transactivating genes transactivated by the corresponding wild-type receptor; contacting the cell

containing the modified receptor protein and exogenous gene with test ligand(s); monitoring expression of the exogenous gene; and identifying the ligand.

As used herein, the modified receptor protein of the invention refers to a receptor protein which has been engineered to be different from the wild-type or unmodified receptor protein. As used herein, the term "modified" is meant to include derivatives of a receptor protein or a ligand in which the amino acid sequence of the protein has been modified in a manner resulting from addition, substitution, insertion or deletion of one or more amino acids in or from the wild type protein. "Modified" may also be used as a general term to denote the modification of any DNA or RNA sequence by addition, substitution, insertion or deletion of one or more nucleotides within that sequence. For example, the nucleic acid encoding the receptor protein can be genetically engineered to include the insertion of nucleotides into the wild-type sequence, deletion of nucleotides from the wild-type sequence, or substitution of nucleotides in the wild-type sequence, for example. Modified receptor proteins of the present invention also include receptors in which the functional domain from one receptor are interchanged with the functional domain from another receptor. Such receptors are referred to as chimeric or hybrid receptors. Chimeric receptors can further be manipulated by the insertion, deletion or substitution of nucleotides.

Receptors are classified into families and superfamilies on the basis of conserved structural features. It is generally believed that under selective pressure for organisms to acquire new biological functions, new receptor family members arose from duplication of existing receptor genes leading to the existence of multi-gene families. Family members thus contain vestiges of the ancestral gene, and these characteristic features can be exploited in the isolation and identification of additional family members. Three of the most well-known receptor superfamilies are the cytokine receptor superfamily, the seven transmembrane domain (7-TMD) receptor superfamily, and the steroid receptor superfamily. The modified receptor proteins of the present invention are preferably steroid receptors.

The steroid receptors may be viewed as ligand-dependent transcription factors. The majority of these receptors appear to contain three domains: a variable, amino-terminal domain; a highly conserved, DNA-binding domain; and a moderately conserved, carboxyl-terminal, ligand-binding domain. The steroid hormone receptor superfamily has been reviewed by Power et al., TIPS 13:318-323, 1992; Parker, Curr. Opin. Cell Biol. 5:499-504, 1993; and McDonnell et al., Bio/Technology 11:1256-1261, 1993. In addition to the known steroid receptors, at least 40 orphan members of this superfamily have been identified (Laudet et al., EMBO J. 11:1003-1013, 1992 and Power et al., ibid.).

Examples of steroid receptors include the vitamin D receptor, glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor, androgen receptor, thyroid receptors, ecdysone receptor, and related estrogen receptors, retinoic acid receptor and retinoid X receptor.

The term "ligand" means a compound that binds in a saturable, high affinity and specific manner to an intracellular protein to elicit a functional response. As used herein, the term "ligand" or "functional ligand" refers to an inducer, such as a hormone or growth substance. For example, ligands include, but are not limited to retinoids.

Inside a cell, the ligand binds to a receptor protein, thereby creating a ligand-receptor complex, which in turn can bind to an appropriate response element (e.g., hormone response element). A single ligand may have multiple receptors. Ligands envisioned for use in the practice of the present invention are characterized as not normally being present in the cell, in other words the ligand is "exogenous" to the cell. An effective amount of ligand contemplated for use in the practice of the present invention is the amount of ligand required to achieve the desired level of gene expression product.

As used herein, the term "DNA-binding domain" of a modified receptor protein, refers to the region of the receptor protein that binds to a hormone response element site on the chromatin DNA. The boundaries for these DNA-binding domains have been identified

and characterized for the steroid hormone superfamily (see US Patent No. 5,171,671, hereby incorporated by reference).

Preferably, the P-box sequence of a receptor DNA-binding domain is modified to have a P-box amino acid sequence that differs from the naturally occurring P-box amino acid sequence. As used herein, the term "P-box" refers to the proximal element region in a DNA-binding domain of a hormone receptor that typically occurs at the junction of the first zinc finger and the linker region, e.g., at about amino acids 19-23 of the DNA-binding domain (see Umesono et al., Cell 57:1139-1146, 1989, Figure 2).

Preferably, the P-box amino acid sequence is modified so that only the half-site nucleotide sequence is recognized by the DNA-binding domain is changed while not altering the spacing between the two half-sites recognized by the DNA-binding domain.

As used herein, the phrase "ligand-binding domain" refers to the region of a receptor protein that binds to ligands such as growth substances or hormones. The boundaries of the ligand-binding domains for the steroid receptor superfamily have been identified and characterized (see US Patent No. 5,171,671).

A "suitable host cell" as used herein refers to a cell into which a nucleic acid encoding an invention modified receptor protein and an exogenous gene functionally linked to a modified response element capable of activation by the modified receptor protein can be introduced and expressed in order to identify a ligand for the modified receptor protein. The invention host cells encompass prokaryotic or eukaryotic cells, preferably eukaryotic. Eukaryotic hosts include yeast (especially Saccharomyces), fungi (especially Aspergillus), mammalian cells (such as, for example, human or primate cells), for use either *in vitro* or *in vivo*.

Yeast and mammalian cells provide substantial advantages in that they can also carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number plasmids which can be utilized for production of the desired proteins in these hosts.

Mammalian cells which may be useful as hosts include cells of fibroblast origin such as VERO or CHO-K1, and their derivatives. For a mammalian host, several possible vector systems are available for the expression of the desired modified receptor protein. A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, etc., may be employed.

To express the desired receptor protein in a prokaryotic cell (such as, for example, *E. coli, B. subtilis, Pseudomonas, Streptomyces*, etc.), it is necessary to operably link the desired receptor protein encoding sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or derepressible).

The desired protein encoding sequence and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a non-replicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the desired ligand molecule may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced sequence into the host chromosome.

An example of a vector that may be employed is one which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may complement an auxotrophy in the host (such as leu2, or ura3, which are common yeast auxotrophic markers), biocide resistance, e.g., antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection.

In a preferred embodiment, the introduced sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Any of a series of yeast gene expression systems can be utilized. Examples of such expression vectors include the yeast 2-micron circle, the expression plasmids YEP13, YCP and YRP, etc., or their derivatives. Such plasmids are well known in the art (Botstein, et al., Miami Wntr. Symp., 19:265-274 (1982); Broach, J. R., In: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 44-470 (1981); Broach, J. R., Cell, 28:203-204 (1982)).

For a mammalian host, several possible vector systems are available for expression. One class of vectors utilize DNA elements which provide autonomously replicating extra-chromosomal plasmids, derived from animal viruses such as bovine papilloma

virus, polyoma virus, adenovirus, or SV40 virus. A second class of vectors relies upon the integration of the desired gene sequences into the host chromosome. Cells which have stably integrated the introduced DNA into their chromosomes may be selected by also introducing one or more markers (e.g., an exogenous gene) which allow selection of host cells which contain the expression vector. The marker may provide for prototropy to an auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper or the like. The selectable marker gene can either be directly linked to the DNA sequences to be expressed, or introduced into the same cell by co-transformation. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama, H., Mol. Cell. Biol., 3:280 (1983), and others.

Once the vector or DNA sequence containing the construct has been prepared for expression, the DNA construct may be introduced (transfect) into an appropriate host. Various techniques may be employed, such as protoplast fusion, calcium phosphate precipitation, electroporation or other conventional techniques. After the fusion, the cells are grown in media and screened for appropriate activities. Binding of ligand to the modified receptor protein results in expression of the exogenous gene sequence.

Typically, nucleic acid sequence information for a desired receptor or other protein can be located in one of several public databases, e.g., Genbank, EMBL, SwissProt, and PIR, or in biological related journal publications. Thus, one of skill in the art would have access to nucleic acid sequence information for virtually all known genes. Those of skill in the art can either obtain the corresponding nucleic acid molecule directly from a public depository or the institution that published the sequence. Alternatively, once the nucleic acid sequence encoding a desired protein has been ascertained, the skilled artisan can employ routine methods, e.g., polymerase chain reaction (PCR) amplification to isolate the desired nucleic acid molecule from the appropriate nucleic acid library. Thus all

known nucleic acids encoding proteins of interest are available for use in the methods and products described herein.

The nucleic acid encoding the modified receptor protein is functionally linked to a modified response element capable of activation by the modified receptor protein wherein the modified receptor protein has a modified ligand-binding domain and a modified DNA binding domain which render it incapable of transactivating genes transactivated by the corresponding wild-type receptor. The term "functionally linked" refers to the nucleic acid sequences in such linkage that they are operational, i.e., the response element can bind with the DNA-binding domain of the modified receptor protein, but not the wild-type receptor protein, and a ligand responsive promoter can control transcription of the exogenous (e.g., reporter or therapeutic) gene, capable of being expressed in the host cell, upon appropriate activation by the response element/receptor protein/ligand complex. "Functionally linked" therefore means that upon appropriate activation, the exogenous gene linked with the other described nucleic acid sequences, is expressed. Expression occurs as the result of a ligand responsive promoter, downstream from the response element, being activated when the response element binds to an appropriate ligand/receptor protein complex. This in turn, then controls transcription of the exogenous gene, which is turned on or otherwise activated as a result of the binding of a ligand/receptor protein complex to the response element.

In the method of the invention, the receptor protein, response element, ligand-binding domain, and DNA-binding domain are all "modified", as discussed above for the modified receptor protein. Thus, the ligand identified by the method of the invention, is incapable of transactivating genes normally transactivated by the wild-type receptor protein. The ligand does not naturally occur in the cell and can specifically transcriptionally modulate expression of the gene encoding the protein of interest (e.g., the exogenous gene). Preferably, the modified ligand-binding domain, the modified DNA-binding domain, and the modified response element do not naturally occur in the host cell. However, a modified receptor protein may consist of a ligand-binding domain

from one naturally occurring receptor and the DNA binding domain of another naturally occurring receptor (i.e., a chimeric receptor). Therefore, the ligand-binding and DNA-binding domains may occur naturally on distinct receptor molecules in the cell.

Modified response elements can be incorporated in multiple copies into various transcription regulatory regions. The modified response elements of the invention can be either a modified wild-type response element, or alternatively, a heterologous response element. Heterologous response elements include elements not naturally occurring in the modified receptor protein (e.g., not naturally occurring in the corresponding wild-type receptor).

One method for identifying and cloning the modified receptor protein ligand genes identified by the method of the present invention, includes screening a library of expression vectors prepared by cloning DNA or cDNA, from a cell capable of expressing such a ligand into an expression vector. The library is then screened for members capable of expressing a protein which binds to the modified receptor protein.

The method of the invention includes contacting the host cell with a test ligand(s). Contacting includes in solution and in solid phase. The test ligand(s) may optionally be a combinatorial library (*e.g.*, chemical combinatorial library) for screening a plurality of ligands. Ligands identified in the method of the invention can be further evaluated and detected, either in solution or after binding to a solid support. Combinatorial chemistry methods for identifying chemical compounds that bind to the receptor are well known in the art.

To determine if a ligand can functionally complex with the receptor protein, induction of the exogenous gene is monitored by monitoring changes in the protein levels of the protein encoded for by the exogenous gene, for example. When a ligand(s) is found that can induce transcription of the exogenous gene, it is concluded that this ligand(s) can

bind to the receptor protein coded for by the nucleic acid encoding the initial sample test ligand(s).

Expression of the exogenous gene can be monitored by a functional assay or assay for a protein product, for example. The exogenous gene is therefore a gene which will provide an assayable/measurable expression product in order to allow detection of expression of the exogenous gene. Such exogenous genes include, but are not limited to, reporter genes such as chloramphenicol acetyltransferase gene, an alkaline phosphatase gene, beta-galactosidase, a luciferase gene, a green fluorescent protein gene, guanine xanthine phosphoribosyltransferase, alkaline phosphatase, and antibiotic resistance genes (e.g., neomycin phosphotransferase).

Expression of the exogenous gene is indicative of ligand-receptor binding, thus, the functional ligand can be identified and isolated. The ligands of the present invention can be extracted and purified from the culture media or a cell by using known protein purification techniques commonly employed, such as extraction, precipitation, ion exchange chromatography, affinity chromatography, gel filtration and the like. Ligands can be isolated by affinity chromatography using the modified receptor protein extracellular domain bound to a column matrix or by heparin chromatography.

The ligand-receptor interaction system described in the method of the invention may utilize the GAL4 protein to provide the DNA binding and transactivation domains (Fields and Song, *Nature*, 340:245, 1989). Other nucleic acid constructs comprising the coding sequences of proteins that have a DNA binding and transactivation domain will be known to those of skill in the art and can be utilized in the method of the invention for providing the hybrid constructs.

Protein ligands identified by the method of the invention include ligands having conservative variations from the originally identified ligands. As used herein, "conservative variations" or "conservative amino acid substitutions" are the substitution

of one amino acid residue in a sequence by another residue of similar properties, such that the secondary and tertiary structure of the resultant peptides are substantially the same. Conservative amino acid substitutions occur when an amino acid has substantially the same charge as the amino acid for which it is substituted and the substitution has no significant effect on the local conformation of the protein. Amino acid pairs which may be conservatively substituted for one another are well-known to those of ordinary skill in the art.

Chemical ligands include naturally occurring structures and variations thereof, including chemical substitutions, as long as the ligand-receptor binding is retained. By providing for the production of large amounts of modified receptor proteins, one can identify ligands or substrates that bind to, modulate or mimic the action of the receptor protein.

Candidate ligands encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate ligands comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate ligands often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate ligands are also found among biomolecules including, but not limited to: peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate ligands are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts

are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological ligands may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, e.g. magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

Ligands identified by the method of the invention can be naturally occurring substances, including antibodies or fragments thereof, chemical molecules, chemically modified substances or synthetic substances, for example. Ligands identified by the method of the invention are preferably "substantially pure", in other words, substantially free of any compound normally associated with the ligand in its natural state (e.g., substantially free of other proteins). The term substantially pure is further meant to describe a ligand of the characteristics used by those of skill in the art. For example, substantially pure ligand proteins will show constant and reproducible characteristics within standard experimental deviations for parameters such as the following: molecular weight, chromatographic techniques, and other such parameters. The term also includes artificial or synthetic mixtures of the ligand and the presence of minor impurities which do not interfere with the biological activity of the ligand, for example, binding to the ligand-binding domain of the modified receptor protein.

In another embodiment, the invention provides a method for modulating the expression of an exogenous gene in a mammalian subject containing a) a DNA construct comprising the exogenous gene under the control of a modified response element; and b)a modified receptor protein which in the presence of a ligand therefor, binds to the modified response element. The method includes administering to the subject an effective amount of a ligand for the modified receptor protein wherein the ligand is not normally present in the cells of the subject and wherein the ligand differs from a ligand that binds to the wild-type receptor.

Exogenous genes, such as therapeutic genes, contemplated for use in the practice of the method of the present invention include genes which encode a gene product which is toxic to the cell in which it is expressed or which imparts a beneficial property to the host subject, and the like. Gene products that induce apoptosis in cells expressing such products are also envisioned for use in the method of the invention.

Any therapeutic agent can be used as the exogenous gene for treatment of a subject as described herein. The term "therapeutic agent" as used herein is meant to refer to any molecule, chemical compound, protein etc. which, when introduced in close association to a cell, is capable of killing, destroying, inhibiting the growth or reproduction of, or otherwise interfering in the normal physiology or metabolism of the cell in a manner not conducive to the cell's survival or reproduction. Examples of suitable therapeutic agents include genes encoding cytotoxic substances, toxins, and the like.

Invention modified receptor proteins can be produced by recombinant methods by introducing an expression construct into appropriate host cells by methods well known in the art. Modified receptor proteins of the invention can then be introduced into a particular subject by direct introduction of the protein, by introducing a DNA construct(s) encoding the receptor into a subject, or by introducing a DNA construct(s) into cells from the subject *ex vivo* and returning the transformed cells to the subject.

In another embodiment of the invention, a modified receptor protein having a modified receptor ligand binding domain and a modified receptor DNA binding domain, wherein the receptor ligand binding domain binds to a ligand different from the wild-type receptor, is also included. A specific modified receptor protein of the invention is a modified retinoic acid receptor protein. Ligands which bind to the modified receptor protein of the invention include retinoids, for example.

Also included in the invention are nucleic acids that encode the modified receptor proteins of the invention. Useful polynucleotide molecules in this regard include mRNA, cDNA and genomic DNA. For recombinant protein production, cDNA is preferred. Methods for preparing these polynucleotide molecules are well known in the art. See, for example, Sambrook et al., eds, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989; Mullis et al., U.S. Pat. No. 4,683,195; and Chirgwin et al., Biochemistry 18: 52-94, 1979. Vectors, enzymes, and other reagents for use in isolation and cloning of polynucleotide molecules are readily available from commercial suppliers.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLES

Modified receptor proteins of the invention are prepared by standard methods. For example, *in vitro* mutagenesis, either directed or random, can be performed on nucleic acid encoding a known receptor protein. Alternatively, a region of a known receptor can be excised and ligated with a region (e.g., DNA-binding region; yeast GAL4) from another receptor to produce a chimeric receptor. In order to assay for a functional ligand for a modified receptor protein, a nucleic acid encoding the modified receptor protein and an exogenous gene functionally linked to a modified response element capable of

activation by the modified receptor protein are transfected into suitable host cells. For example, HeLa CV1 293 or 3T3 fibroblasts cells are transfected with a gene encoding a modified retinoic acid receptor functionally linked to a reporter gene (*e.g.*, if GAL4 is used as the DNA binding domain, Upstream Activated Sequences GAL4 is used). A binding site, UAS, TK promoter, a luciferase gene may be employed, for example. Suitable host cells will be modified receptor-deficient. Conditions for growth and transfection of the host cells are standard in the art.

As those skilled in the art will appreciate, various constructs containing nucleic acids encoding modified receptor proteins and reporter genes suitable for assaying ligand-/receptor binding in the method of the invention can be constructed.

The transfected cells are systematically challenged/contacted with a series of candidate or test ligands and binding is monitored by a detectable assay. For example, the test ligand(s) may be part of a combinatorial library. Reporter genes used as the exogenous gene in this assay are described herein in the specification; others will be known to those of skill in the art. Once expression of the reporter/exogenous gene is detected, the ligand can be identified, for example, by isolating from a chemical combinatorial library.

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

CLAIMS

1. A method for identifying a functional ligand for a modified receptor protein, said method comprising:

   a) introducing into a suitable host cell, a nucleic acid encoding a modified receptor protein and an exogenous gene functionally linked to a modified response element capable of activation by the modified receptor protein wherein the modified receptor protein has a modified ligand-binding domain and a modified DNA binding domain which render it incapable of transactivating genes transactivated by the corresponding wild-type receptor;

   b) contacting the cell containing the modified receptor protein and exogenous gene with test ligand(s);

   c) monitoring expression of the exogenous gene; and

   d) identifying the ligand.

2. The method of claim 1, wherein the receptor protein is selected from the group consisting of retinoic acid receptors, glucocorticoid receptor, mineralocorticoid receptor, thyroid receptors, ecdysone receptor, and estrogen related receptors.

3. The method of claim 1, wherein the host cell is a mammalian cell.

4. The method of claim 1, wherein the host cell is a yeast cell.

5. The method of claim 1, wherein the modified receptor binds to a ligand not capable of activating the wild-type receptor.

6. The method of claim 1, wherein the modified ligand-binding domain contains at least one amino acid modification.

7. The method of claim 6, wherein the modification is a deletion, a substitution or an insertion in the polynucleotide encoding the ligand-binding domain.

8. The method of claim 1, wherein the exogenous gene is selected from the group consisting of a chloramphenicol acetyltransferase gene, an alkaline phosphatase gene, a luciferase gene, a green fluorescent protein gene, and an antibiotic resistance gene.

9. The method of claim 1, wherein the response element is a modified wild type response element or a heterologous response element.

10. The method of claim 1, wherein test ligand(s) is a combinatorial library.

11. The method of claim 1, wherein said contacting is carried out in solution.

12. The method of claim 1, wherein said contacting is carried out in solid phase.

13. The method of claim 1, wherein the DNA binding domain is characterized as having a P-box amino acid sequence that differs from the P-box amino acid sequence of the corresponding wild-type DNA binding domain.

14. The method of claim 13, wherein the P-box amino acid sequence of the modified receptor protein preferentially binds to a response element that differs from the response element to which the corresponding wild-type P-box amino acid sequence binds.

15. TMOC 13, wherein the DNA binding domain is GAL4.

16. A functional ligand identified by the method of claim 1.

17. A method for modulating the expression of an exogenous gene in a mammalian subject containing:

a) a DNA construct comprising said exogenous gene under the control of a modified response element; and

b) a modified receptor protein which in the presence of a ligand therefor, binds to said modified response element;

said method comprising administering to said subject an effective amount of a ligand for said modified receptor protein; wherein said ligand is not normally present in the cells of the subject and wherein said ligand differs from a ligand that binds to the wild-type receptor.

18. The method of claim 17, wherein said modified response element has no binding affinity for the wild-type receptor protein.

19. The method of claim 17, wherein the modified receptor protein has a modified ligand-binding domain and a modified DNA binding domain as compared with the corresponding wild-type receptor.

20. The method of claim 17, wherein the modified receptor binds to a ligand not capable of activating the wild-type receptor.

21. The method of claim 17, wherein the modified ligand-binding domain contains modification in at least one amino acid.

22. The method of claim 21, wherein the modification is selected from the group consisting of a deletion, a substitution and an insertion in the polynucleotide encoding the ligand-binding domain.

23. The method of claim 17, wherein the response element is a modified wild type response element or a heterologous response element.

24. The method of claim 19, wherein the DNA binding domain of the modified receptor protein is characterized as having a P-box amino acid sequence different from the P-box amino acid sequence of the corresponding wild-type DNA binding domain.

25. The method of claim 24, wherein the P-box amino acid sequence of the modified receptor protein binds to a response element different from the response element to which the corresponding wild-type P-box amino acid sequence binds.

26. The method of claim 17, wherein said exogenous gene is a wild type gene and/or a therapeutic gene.

27. The method of claim 27, wherein said exogenous gene encodes a toxin.

28. A modified receptor protein comprising:
a modified receptor ligand binding domain and a modified receptor DNA binding domain, wherein the receptor ligand binding domain binds to a ligand different from the wild-type receptor.

29. A modified retinoic acid receptor protein comprising:
a modified retinoic acid receptor ligand binding domain and a modified retinoic acid receptor DNA binding domain, wherein the modified retinoic acid receptor ligand binding domain binds to a ligand different from the wild-type retinoic acid receptor.

30. A nucleic acid sequence encoding the receptor of claim 28 or 29.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10671

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/00; C12N 15/11; G01N 33/53
US CL :435/7.2; 530/350; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2; 530/350; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- A	SCHULMAN et al., Interactions between the retinoid X receptor and a conserved region of the TATA-binding protein mediate hormone-dependent transactivation. Proc. Natl. Acad. Sci. USA. August 1995. Vol. 92, pages 8288-8292, especially figures 2 and 4.	1-4, 6-7, 9, 11, 13, 15 -------- 5, 8, 10, 12, 14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
08 AUGUST 1998

Date of mailing of the international search report
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10671

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-15

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10671

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, BIOSCIENCE, MEDLINE, BIOSIS, CAPLUS

search terms: ligand binding domain, DNA binding domain, nuclear receptor, mutagenesis

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, Claims 1-15, drawn to a method for identifying a functional ligand.
Group II, Claim 16, drawn to a functional ligand.
Group III, Claims 17-27, drawn to a method for modulating the expression of an exogenous gene by administering a ligand.
Group IV, Claims 28-30, drawn to a modified receptor protein and a nucleic acid sequence encoding the receptor protein.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features because the method of claim 1 is anticipated by SCHULMAN et al. and thus does not share a special technical feature with any other group.

Group I does not share special technical feature with any other group because the claimed compounds are not novel as evidenced by SCHULMAN et al.

The product of group II does not share special technical feature with the product of group VI, because each defines a separate invention over the art. The products are drawn to products having materially different structures and functions.

The method of group I does not share special technical feature with the method of group III, because each defines a separate invention over the art. The methods of inventions I and III, are drawn to processes having materially different process steps, which are practiced for materially different purposes and each defines a separate invention over the art.